LIPOSOME FOR INCORPORATING LARGE AMOUNTS OF

HYDROPHOBIC SUBSTANCES

This application is a continuation-in-part of Ser. No. 09/866,584 filed

May 30, 2001, now abandoned.

5   BACKGROUND OF THE INVENTION

Field of the Invention

[0001]   The invention relates to the use of liposomes in the drug delivery

system, and more particularly to the stable liposomes capable of incorporating

high content of hydrophobic drugs.

10   Description of the Related Art

[0002]   Liposome technology has been exploited extensively for the

purpose of drug delivery for many years.   A typical liposome structure is

composed of single or multiple layer membranes with hydrophobic domain

between the phospholipid bilayers, and the interior aqueous compartment.

15   Hydrophobic or hydrophilic compounds can be entrapped in the hydrophobic

domain or encapsulated in the aqueous compartment, respectively.   On the

other hand, liposomes can be constructed of natural constituents so that the

liposome membrane is in principal identical to the lipid portion of natural cell

membranes.   It is considered that liposomes are quite compatible with the

20   human body when used as drug delivery system.   In addition,

liposome-based drug formulation also has been reported to be able to achieve the equivalent therapeutic efficacy to free drug, as well as reduce the systemic toxicity in many applications.

[0003]   The hydrophobic drug, paclitaxel, was sold in the market in 1992, and used in phase II trials for treating breast and ovarian cancer.   In 1998, it was used in combination therapy with cisplatin for the treatment of non-small cell lung and ovarian cancer in phase I trials.   However, due to its poor solubility in water, paclitaxel is prepared for clinical administration containing Cremophor EL® (polyethoxylated castor oil) and absolute ethanol in a 50/50 (vol/vol) ratio (Diluent 12).   In clinical trials, the problems of anaphylactoid reaction, neutropenia, peripheral neuropathy, bradyarrhythmia and anemia were encountered.   Meanwhile, the amount of cremophor EL necessary to solubilize the clinically required dose of paclitaxel is much higher than that administered with any other marketed drug.   Cremophor vehicle thus is found to be responsible for hypersensitivity response.   Premedication with corticosteroid, diphenhydramine or H_2 antagonist, and slow infusion of a large volume are needed to avoid the side effect.   In contrast, owing to the aforementioned advantages of liposome-based drug delivery system, researches of incorporating paclitaxel in liposomes for clinical paclitaxel administration have become a hot topic and been reported regularly.

[0004]   Conventional paclitaxel-liposomes were prepared at paclitaxel/lipid molar ratio of approximately 3 mole% regardless of whether the liposomes are made of a mixture of phosphatidyl glycerol (PG) and phosphatidyl choline

(PC) (USP 5,415,869; Sampedro, F et al., *J Micrencapsul* 11:309-318 (1993);

Sharma, A. et al., *Pharm Res* 11:889-896 (1994); Shien, M.F. et al., *J Ferm*

Bioeng 83:87-90 (1997)), or of unsaturated (USP 6,090,955; Bartoli, M.H. et

al., *J Micrencapsul* 7:191-197 (1990); Riondel, J. et al., *In Vivo* 6:23-28 (1992);

5   Sharma, D. et al., *Melanoma Res* 8:240-244 (1998)) or partially unsaturated

PC (USP 5,683,715).   At a drug/lipid ratio of 4 mole %, the

paclitaxel-liposome system is stable only for 2 days while needle-like crystal

precipitates appear during preparation at a drug/lipid ratio up to 8 mole %

(Sathyamangalam, V. et al., *Biochemistry* 33:8941-8947 (1994); Bernsdorff, C.

10   et al., *J Biomed Mater Res* 46:141-149 (1999)).   On the other hand, the

liposomes are prepared by employing hydrophilic polymer-conjugated

phospholipid (methoxy polyethylene glycol-phosphatidyl ethanolamine) in

order to enhance its circulation time in blood post iv administration (Crosasso,

P. et al., *J Control Release* 63:19-30 (2000)).   Liposomes with the prolonged

15   circulation time in bloodstream make it possible increasing the availability of

the injected liposomes to reach the target cells before being metabolized.

However, this formulation of the polymer-engrafted liposomes with a maximal

3 mole % (paclitaxel/lipid ratio) quickly become unstable in one-week storage

at 4°C.

20   [0005]   Alternatively, a formulation of paclitaxel-liposomes comprising a

special phospholipid, cardiolipid, and phosphatidyl choline (PC) was disclosed

in USP 5,424,073 and *Int J Oncol* 12:1035-1040 (Cabanes, A. et al., 1998).

The molecular structure of cardiolipid is composed of one huge hydrophilic

head and four aliphatic chains.   The liposomes prepared in accordance with this formulation increase the paclitaxel/lipid molar ratio to 9 mole%, however, it is stable only for 1 month when stored in liquid form at 4°C.

[0006]   Generally, paclitaxel incorporated within the bilayer membrane of liposomes is thermodynamically prone to self-aggregation, then precipitating from liposomes. Previous researches have reported that the optimal paclitaxel /lipid molar ratio in a typical liposome formulation is ranged from 3 to 4 mole %, and paclitaxel-liposomes are more stable when the drug/lipid ratio is kept at approximately 3 mole %.   When the molar ratio is increased, needle-like crystal precipitates appear during the preparation process.   Besides, it is known by person skilled in the art that drugs with a low drug/lipid ratio are commonly unsuitable for clinical administration.   A high dose of liposomes still may result in certain extent of toxicity due to the injection of excessive amounts of lipids in the body.   Furthermore, increasing liposome concentration also raises the cost of production.   Therefore, it is important to elevate the hydrophobic drug/lipid ratio in liposome-based drug delivery system by which the above drawbacks may be avoided.

SUMMARY OF THE INVENTION

[0007]   It is therefore an object of the invention to provide a liposome-based drug delivery system that is able to incorporate large amounts of hydrophobic compounds.   Accordingly, the formulated liposomes capable of incorporating high content of hydrophobic compounds can

maintain considerably stable for months, as well as reduce the possible side effects in the versatile applications.

[0008]   The invention achieves the above-identified objects by providing the formulated liposomes for incorporating large amount of hydrophobic

5   compounds.   The composition of the liposomes at least comprises a first and second phospholipids, hydrophobic drugs and other additives such as lipids modified by hydrophilic polymer (such as MPEG-DSPE), cholesterol, cholesterol derivatives and antioxidants.   According to the invention, the phase transition temperature of the first phospholipids, T_{g1}, is in the range

10   from 40 to 70 °C, and preferably from 50 to 65 °C.   The phase transition temperature of the second phospholipids, T_{g2}, is in the range from −30 to 20 °C, and preferably from −20 to 4 °C.   Also, the phase transition temperature of the first phospholipids, T_{g1}, is higher than that of the second phospholipids, T_{g2} while the drug delivery temperature T_1 and storage temperature T_2 are

15   chosen at specified ranges subject to the order of $T_{g1} > T_1 > T_2 > T_{g2}$.   The first phospholipids having higher phase transition temperature forms the gel state phase, and the second phospholipids having lower phase transition temperature forms the liquid-crystal phase.   Each membrane lipid bilayer consists of several regions of gel state phases and liquid-crystal phases, and

20   the hydrophobic drugs can be held within the lipid bilayer.   The phase boundary barrier between the regions of gel state phase and liquid-crystal phase is able to reduce lateral movement and aggregation of the hydrophobic drugs, thereby stabilizing the liposomes.   Thus, this liposome composition

results in coexistence of multiple discontinuous immiscible phases (gel state

phase and liquid-crystal phase) occurring on each bilayer membrane of

liposomes regardless of unilamellar or mulitlamellar structure when the drug is

delivered or stored.   The drug delivery temperature T_1 is optionally from 30 to

5   38 °C, while the storage temperature T_2 is optionally from 4 to 25 °C.

[0009]   The first phospholipids, with higher phase transition temperature

(T_{g1}) from 40 to 70 °C, are preferably hydrogenated naturally-occurring

phospholipids and saturated phospholipids with long carbon chain (-$(CH2)_n$-,

the value of n is at least 14), such as phosphatidyl choline (PC), phosphatidyl

10   glycerol (PG), phosphatidyl serine (PS), phosphatidyl acid (PA), or

phosphatidyl ethanolamine (PE).   Examples of hydrogenated phosphatidyl

choline (PC) are hydrogenated egg phosphatidyl choline (HEPC) (T_g = 50~55

°C) and hydrogenated soy phosphatidyl choline (HSPC) (T_g = 55 °C), while

examples of saturated phsopholipids with long carbon chains (-$(CH2)_n$-, the

15   value of n is at least 14) are dipalmitoyl phosphatidyl choline (DPPC) (T_g = 42

°C), distearyloyl phosphatidyl choline (DSPC) (T_g = 55 °C), diarachidoyl

phosphatidyl choline (Tg = 66 °C), dimyristoyl phosphatidyl ethanolamine

(DMPE) (Tg = 49.5 °C), dipalmitoyl phosphatidyl ethanolamine (DPPE) (Tg =

64 °C), distearoyl phosphatidyl ethanolamine (DSPE) (Tg = 74 °C),

20   diarachidoyl phosphatidyl ethanolamine (Tg = 82 °C), dipalmitoyl phosphatidyl

glycerol (DPPG) (Tg = 41.5 °C), distearoyl phosphatidyl glycerol (Tg = 54.5

°C), dimyristoyl phosphatidyl acid (DMPA) (Tg = 50 °C), dipalmitoyl

phosphatidyl acid (DPPA) (Tg = 66 °C), dipalmitoyl phosphatidyl serine (DPPS)

(Tg = 54 °C), and distearoyl phosphatidyl serine (DSPS) (Tg = 70 °C). The desired phospholipids may also be a combination of two or more phospholipids listed above.   The lists of PC above are illustrations of specific phospholipids but are in no way intended to limit the scope thereof.

5   [0010]   The second phospholipids, with lower phase transition temperature (T_{g2}) from −30 to 10 °C, are preferably unsaturated phospholipids or saturated phospholipids with short carbon chains (-$(CH2)_n$-, the value of n is at most 14), such as phosphatidyl choline (PC), phosphatidyl glycerol (PG), phosphatidyl serine (PS), phosphatidyl acid (PA), or phosphatidyl ethanolamine (PE).

10   Examples of synthetic or naturally-occurring unsaturated phospholipids are egg phosphatidyl choline (EPC) (T_g = -8 °C) and soy phosphatidyl choline (SPC) (T_g = 0 °C), oleoyl palmitoyl phosphatidyl choline (Tg = -10 °C), dioleoyl phosphatidyl choline (Tg = -19 °C), dipetroselinoyl phosphatidyl choline (Tg = 1 °C), dipalmitelaidoyl phosphatidyl choline (Tg = -4 °C), dipalmitoleoyl

15   phosphatidyl choline (Tg = -36 °C), dipalmitelaidoyl phosphatidyl ethanolamine (Tg = -33.5 °C), dioleoyl phosphatidyl ethanolamine (Tg = -16 °C), dioleoyl phosphatidyl serine (Tg = -10 °C), while examples of synthetic or naturally-occurring saturated phospholipids with short carbon chains is dilauroyl phosphatidyl choline (DLPC) (T_g = -2 °C). diundecanoyl phosphatidyl

20   choline (Tg = -15.5 °C), didecanoyl phosphatidyl choline (Tg = -34.7 °C), dinonanoyl phosphatidyl choline (Tg = -55.2 °C), didecanoyl phosphatidyl ethanolamine (Tg = 3.6 °C), dinonanoyl phosphatidyl ethanolamine (Tg = -14.5 °C).   The desired phospholipids may also be a combination of two or

more phospholipids listed above.   The list of phospholipids above are

illustrations of specific phospholipids but are in no way intended to limit the

scope thereof.

[0011]   Other objects, features, and advantages of the invention will

5   become apparent from the following detailed description of the preferred but

non-limiting embodiments.   The following description is made with reference

to the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

[0012]   FIG. 1 illustrates that coexisting phenomenon of two phases on the

10   bilayer membrane of liposomes resulting from the liposomes composed of

two phospholipids with very different phase transition temperatures.

[0013]   FIG. 2 illustrates that the incorporation efficiency and particle size of

liposomes are affected by altering cholesterol content according to a

preferred embodiment of the invention.

15   [0014]   Table 1 shows incorporation efficiency of liposomes with different

paclitaxel/lipid molar ratios, according to a preferred embodiment of the

invention.

[0015]   Table 2 shows incorporation efficiencies of the liposomes

composed of only unsaturated or saturated phosphatidyl cholines at different

20   drug/lipid molar ratios according to a preferred embodiment of the invention.

[0016]   Table 3 shows the shelf stability of liposomes at different drug/lipid

molar ratios according to a preferred embodiment of the invention.

[0017]   Table 4 shows incorporation efficiency and particle size of different

liposome compositions, according to a preferred embodiment of the invention.

5   [0018]   Table 5 shows survival rate of mice received i.v. injections of either

conventional paclitaxel (Cremophore EL/Ethanol = 50/50 (v/v)) or

paclitaxel-liposomes at doses of 20 and 40 mg/kg, according to a preferred

embodiment of the invention.

DETAILED DESCRIPTION OF THE INVENTION

10   [0019]   It is disclosed in the invention that at special ranges of two

phospholipid combination and temperature, liposome composed of two

phospholipids such as an unsaturated phospholipid (the second phospholipid)

and a saturated phospholipid (the first phospholipid) with different phase

transition temperatures are able to form two separated phases, a gel state

15   phase and liquid-crystal phase, in the phospholipid bilayer, as shown in Fig.1.

The two immiscible phases coexist in the liposomes and create several

discontinuous regions.   The first phospholipid having higher phase transition

temperature forms the gel state phase, and the second phospholipid having

lower phase transition temperature forms the liquid-crystal phase.   Each

20   membrane bilayer consists of several regions of gel state phases and

liquid-crystal phases, and the hydrophobic compounds can be held within the

lipid bilayer.   The phase boundary barrier between the regions of gel state phase and liquid-crystal phase is able to reduce lateral movement and aggregation of the hydrophobic compounds, thereby stabilizing the liposome.

[0020]   It has been reported that hydrophobic compounds such as

5   paclitaxel has a tendency to undergo concentration-dependent aggregation in hydrophobic environment, forming intermolecular hydrogen bonds (Sathyamanglam, V. et al., *J Pharm Sci* 83: 1470-76 (1994)).   Similarly, as a large amount of paclitaxel was embedded in the hydrophobic domain within bilayer membrane, it is thermodynamically prone to self-aggregating,

10   destablizing the liposomes.   Accordingly, when the formulated liposomes are prepared, two immiscible phases are formed and phase boundaries are speculated to construct a barrier stopping the self-aggregation process of hydrophobic molecules.   As a result, stable liposomes capable of incorporating high content of hydrophobic compound become possible.   The

15   existence of lateral phase-separated phospholipid-regions is advantageous for incorporating large amount of hydrophobic molecules into the phospholipid bilayer.   The formulated liposomes can incorporate higher content of paclitaxel and remain more stable than any other liposome formulations ever reported.

20   [0021]   The invention, hence, provides a liposome-based drug delivery system composing of two phospholipids with different phase transition temperatures.   The phospholipids with high (T_{g1}) and low (T_{g2}) phase transition temperatures can be saturated and unsaturated phospholipids,

respectively.   The coexistence of several discontinuous immiscible phases (e.g. gel phase and liquid-crystal phase) occurs in phospholpiid bilayer at a specific phospholipid composition and temperature (T), wherein $T_{g1} > T > T_{g2}$. The specific temperature T can be the liposome delivery temperature T_1

5   (about 30~38°C) or storage temperature T_2 (about 4~25°C).   Therefore, T is

commonly considered as a range that includes the liposome delivery and storage temperatures.   The temperature range provides the requirement to design the particular combination of two phospholipids to achieve phase separation.   For example, when the intravenous injection is administrated

10   (intravenous administration temperature is 37 °C (T_1) and liposomes are stored at is 4 °C (T_2)), a phospholipid with phase transition temperature larger than 40 °C ($T_{g1} > 40$ °C) could be carefully chosen as the first phospholipid, and preferably with phase transition temperature ranged from 40 to 70 °C. Also, a phospholipid with phase transition temperature lower than 4 °C ($T_{g2} <$

15   4 °C) could be carefully chosen as the second phospholipid, and preferably in a range from –30 to 4 °C.   When the subcutaneous injection is administrated (administration temperature is 32 °C (T_1) and liposomes are stored at is 25 °C (T_2)), a phospholipid with phase transition temperature larger than 35 °C ($T_{g1} > 35$ °C) could be carefully chosen as the first phospholipid, and preferably

20   with phase transition temperature ranged from 35 to 60 °C.   Also, a phospholipid with phase transition temperature lower than 25 °C ($T_{g2} < 25$ °C) could be carefully chosen as the second phospholipid, and preferably in a range from –20 to 10 °C.

[0022]   The first phospholipids, with higher phase transition temperature (T_{g1}) from 40 to 70 °C, are preferably hydrogenated naturally-occurring phospholipids and saturated phospholipids with long carbon chain ($-(CH2)_n-$, the value of n is at least 14), such as phosphatidyl choline (PC), phosphatidyl glycerol (PG), phosphatidyl serine (PS), phosphatidyl acid (PA), or phosphatidyl ethanolamine (PE).   Examples of hydrogenated phosphatidyl choline (PC) are hydrogenated egg phosphatidyl choline (HEPC) (T_g = 50~55 °C) and hydrogenated soy phosphatidyl choline (HSPC) (T_g = 55 °C), while examples of saturated phsopholipids with long carbon chains ($-(CH2)_n-$, the value of n is at least 14) are dipalmitoyl phosphatidyl choline (DPPC) (T_g = 42 °C), distearyloyl phosphatidyl choline (DSPC) (T_g = 55 °C), diarachidoyl phosphatidyl choline (Tg = 66 °C), dimyristoyl phosphatidyl ethanolamine (DMPE) (Tg = 49.5 °C), dipalmitoyl phosphatidyl ethanolamine (DPPE) (Tg = 64 °C), distearoyl phosphatidyl ethanolamine (DSPE) (Tg = 74 °C), diarachidoyl phosphatidyl ethanolamine (Tg = 82 °C), dipalmitoyl phosphatidyl glycerol (DPPG) (Tg = 41.5 °C), distearoyl phosphatidyl glycerol (Tg = 54.5 °C), dimyristoyl phosphatidyl acid (DMPA) (Tg = 50 °C), dipalmitoyl phosphatidyl acid (DPPA) (Tg = 66 °C), dipalmitoyl phosphatidyl serine (DPPS) (Tg = 54 °C), and distearoyl phosphatidyl serine (DSPS) (Tg = 70 °C).   The desired phospholipids may also be a combination of two or more phospholipids listed above.

[0023]   The second phospholipids, with lower phase transition temperature (T_{g2}) from −30 to 10 °C, are preferably unsaturated phospholipids or saturated

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phospholipids with short carbon chains ($-(CH2)_n-$, the value of n is at most 14),

such as phosphatidyl choline (PC), phosphatidyl glycerol (PG), phosphatidyl

serine (PS), phosphatidyl acid (PA), or phosphatidyl ethanolamine (PE).

Examples of synthetic or naturally-occurring unsaturated phospholipids are

5   egg phosphatidyl choline (EPC) (T_g = -8 $^{\circ}$C) and soy phosphatidyl choline

(SPC) (T_g = 0 $^{\circ}$C), oleoyl palmitoyl phosphatidyl choline (Tg = -10 $^{\circ}$C), dioleoyl

phosphatidyl choline (Tg = -19 $^{\circ}$C), dipetroselinoyl phosphatidyl choline (Tg =

1 $^{\circ}$C), dipalmitelaidoyl phosphatidyl choline (Tg = -4 $^{\circ}$C), dipalmitoleoyl

phosphatidyl choline (Tg = -36 $^{\circ}$C), dipalmitelaidoyl phosphatidyl

10   ethanolamine (Tg = -33.5 $^{\circ}$C), dioleoyl phosphatidyl ethanolamine (Tg = -16

$^{\circ}$C),dioleoyl phosphatidyl serine (Tg = -10 $^{\circ}$C), while examples of synthetic or

naturally-occurring saturated phospholipids with short carbon chains is

dilauroyl phosphatidyl choline (DLPC) (T_g = -2 $^{\circ}$C). diundecanoyl phosphatidyl

choline (Tg = -15.5 $^{\circ}$C), didecanoyl phosphatidyl choline (Tg = -34.7 $^{\circ}$C),

15   dinonanoyl phosphatidyl choline (Tg = -55.2 $^{\circ}$C), didecanoyl phosphatidyl

ethanolamine (Tg = 3.6 $^{\circ}$C), dinonanoyl phosphatidyl ethanolamine (Tg =

-14.5 $^{\circ}$C).   The desired phospholipids may also be a combination of two or

more phospholipids listed above.

[0024]   The following examples illustrate methods of preparing hydrophobic

20   drugs/liposomes with phospholipids with high or low transition temperatures.

The examples are intended to illustrate specific liposome compositions that

include the phospholipids listed above, and methods of the invention, but are

in no way intended to limit the scope thereof.

Example 1

[0025]   A method of preparing paclitaxel-liposomes.

[0026]   In order to prepare a paclitaxel (added amounts)/lipid molar ratio of

1/14, 1.23 mg paclitaxel was added into the alcoholic admixture of the second

5   phospholipid-12.2 mg/ml egg phosphatidyl choline (EPC), the first

phospholipid- 2.28 mg/ml hydrogenated egg phosphatidyl choline (HEPC),

and other additives- 2.28 mg/ml cholesterol and 5.4 mg/ml methoxy

polyethylene glycol-distearyloyl phosphatidyl ethanolamine (MPEG-DSPE).

The alcoholic admixture may also contain other antioxidants and cholesterol

10   or cholesterol derivatives.   Therefore, the composition of alcoholic admixture

illustrated in this example was not to be limited.   The solution was

evaporated under vacuum to remove the solvent and form a lipid film on the

wall of the round-bottom flask at which time, 1 ml, 10 % (w/v) sucrose was

added to the flask for hydration.   Large multilamellar liposomes were

15   suspended, followed by sonicating for 10 mins in order to obtain small

unilamellar vesicles.   Paclitaxel-containing liposomes were then sterilized by

filtration through 0.2 μm cellulose acetate membrane.   Laser particle size

analyzer (Coulter N4 plus) was used to analyze the particle sizes of the

vesicles. The average diameter was approximately 120 nm.   After filtration,

20   the concentration of the incorporated paclitaxel in the liposome is determined

by HPLC.   It was approximately 1.0 mg/ml and the incorporation efficiency

was about 80%.

[0027]   In Example1, hydrogenated egg phosphatidyl choline (HEPC)

selected as the first phospholipid has a phase transition temperature of 50~55

$^{\circ}$C which is higher than the intravenous administration temperature (37 $^{\circ}$C)

and the storage temperature (4 $^{\circ}$C).   Egg phosphatidyl choline (EPC)

5   selected as the second phospholipid has a phase transition temperature of -8

$^{\circ}$C which is lower than the intravenous administration temperature (37 $^{\circ}$C)

and the storage temperature (4 $^{\circ}$C).

Example 2

10   [0028]   A method of preparing paclitaxel-liposomes.   Procedure is similar

to example 1 in addition to extrusion unit.

[0029]   Preparation of the liposomes with the addition of extrusion unit was

similar to that in the Example 1.   Whereas, the liposomes obtained from

sonication or hydration units were followed by extrusion with a series of

15   membranes.   Polycarbonate membranes with a uniform pore size ranging

from 1.0 to 0.2 μ m were used.   The pressure from a nitrogen tank provided

the driving force.   The maximum pressure was set up to 750 psi according to

the operation manual of the equipment manufactured by Lipex Co.   The

resultant liposome sample was sterilized by filtration, too.   In this way, a

20   narrow distribution of particle size of liposomes was obtained; the average

particle size was estimated to be 150 nm with polydispersity index of 0.3.

The loss of the incorporated paclitaxel during the process was about 10 %.

Example 3

5   **[0030]**   Effect of increasing drug/lipid ratio

[0031]   Various aliquots of 10 mg/ml paclitaxel were added into the

admixture to change the drug/lipid molar ratio in the liposomes.   The method

of preparing liposomes was similar to the one described in Example 1.   The

results are listed in Table 1.   Incorporation efficiency of paclitaxel was

10   calculated just after preparation according to the concentration determination

by HPLC.   It was found (Table 1) that incorporation efficiency was maintained

above 80 % as the added paclitaxel/lipid molar ratio increases up to 20%.

However, incorporation efficiency dropped to 60 % when the added paclitaxel

/lipid ratio was increased to 25 mole %.

15   **[0032]**   According to these experiments, it is proved that the two immiscible

phases (with several discontinuous regions as shown in Fig. 1) coexisting in

the liposomes are capable of prevent the self-aggregation of hydrophobic

drugs, thereby greatly increasing the amounts of drug held stably within the

bilayer of the liposomes.   For example, the incorporation amounts (molar %)

20   of paclitaxel had been raised up to 17 mole% when the formula G (Table 1) is

conducted.   Also, no precipitates appeared during liposome preparation.

Moreover, the lipid concentration has no effect on the incorporation amounts

(molar %) of paclitaxel (comparison the results of formula B and formula C).

Table 1

Formula	Lipid Conc. (mM)	paclitaxel Added Amount/Lipid (mole %)	Incorporated paclitaxel (mg/ml)	Incorporation Efficiency (%)	Incorporation Amounts (mole %)	Particle Size Average ± SD (nm)
A*	40	3	1.03	80.4	2.4	120.0 ± 45.5
B#	20	7	1.04	84.5	5.9	114.3 ± 43.6
C#	40	7	2.02	82.4	5.8	115.8 ± 41.0
D#	20	10	1.34	78.8	7.9	116.2 ± 44.1
E#	20	13	1.60	75.0	9.8	119.0 ± 44.2
F#	20	15	2.07	81.0	12.2	125.4 ± 46.8
G#	20	20	2.90	85.1	17.0	134.9 ± 44.6
H#	20	25	2.32	54.6	10.9	146.3 ± 50.4

* Compositions of liposome - second phospholipid EPC : first phospholipid HEPC : other additives (cholesterol/MPEG) = 32 : 8 : (12/2).

Compositions of liposome - second phospholipid EPC : first phospholipid HEPC : other additives (cholesterol/MPEG) = 16 : 3 : (6/2).

Paclitaxel Incorporation Amounts (molar %) = Paclitaxel Added Amounts (molar ratio)×Incorporation Efficiency (%).

Comparative Example 1

[0033]   Comparison with liposomes made of phospholipids with either high or low transition temperature.

[0034]   The liposomes composed of only one unsaturated or saturated phosphatidyl cholines (PC) were prepared by the method with similar procedures as described in Example 1.   The results were listed in Table 2 and showed that the liposomes made of egg PC was able to incorporate more than 90% paclitaxel only when the added paclitaxel/lipid molar ratio was kept

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at 3 mole%.   However, incorporation efficiency dropped to 40 % once the

molar ratio was raised up to 7 mole%.   If the added paclitaxel/lipid molar ratio

was raised up to 7 mole%, the incorporation efficiency dropped to 40 %.

[0035]   Also, the liposomes made of only HEPC could not incorporate more

5   than 3 mole% of paclitaxel.   The incorporation efficiency was estimated to be

40-60% when the added paclitaxel/lipid ratio was 3 mole%.

[0036]   Compare the results (Table 2) with Example 3 (Table 1), it indicated

that the liposomes made of the phospholipids only with either high or low ·

transition temperature can not achieve the object of incorporating high

10   content of hydrophobic drugs.   The paclitaxel incorporation amount of the

liposomes mostly made of EPC is not more than 3 mol% (Table 2).   The

paclitaxel incorporation amounts of the liposomes mostly made of HEPC is

only about 2 mol% (Table 2).   Moreover, the paclitaxel incorporation amounts

is 1.0 mol% when two phospholipids, HEPC (T_g = 50~55°C) and DPPG (T_g =

15   41°C) both with high transition temperatures, were selected as the first and

second phospholipids of liposomes.

Table 2

Liposome Composition (molar ratio)				Lipid Conc. (mM)	Paclitaxel Added Amount /Lipid (mole %)	Incorporated paclitaxel (mg/ml)	Incorporation Efficiency (%)	Incorporation Amounts (mole %)	Particle Size Average± SD (nm)
Second Phospho -lipid	First Phospho -lipid	Other Additives							
EPC	HEPC	Cholesterol	MPEG						

20	-	8	1	20	3	0.45	88.4	2.7	142.9± 55.4
20	-	8	1	20	7	0.52	42.1	2.9	174.1 ± 71.3
-	10	1	1	20	3	0.35	68.1	2.0	3.2 ± 36.1
-	10	1	1	20	3	0.35	68.1	2.0	93.2 ± 36.1
DPPG	HEPC	Cholesterol	MPEG						
3	7	1	-	60	3	1.20	34.0	1.0	118.9 ± 45.2

Example 4

5 [0037] Stability of paclitaxel-liposome stored at 4°C.

[0038] Paclitaxel-liposomes prepared by different molar ratio of liposome

compositions (listed in Table 3) were stored at 4°C immediately after

liposome-forming process. Paclitaxel crystals and liposomes with large

particle sizes were removed by filtration through 0.2 μm CA-membrane. The

10 concentration of paclitaxel was determined by HPLC. The results were listed

in Table 3. According to these experiments of Example 4, it has disclosed

that the liposomes comprising EPC and HEPC and added large amount of

paclitaxel are greatly stable, and are even stable after 6-month storage.   As

indicated in Table 4, the incorporation efficiency of the liposomes, originally

15 having the added paclitaxel/lipid ratio of 20 mole %, had dropped to 69% of

initial incorporation efficiency after 6-month storage. However, the

incorporation efficiency of the liposomes, that originally has the added

paclitaxel/lipid ratio of 15 mole%, had been at over 85% of initial incorporation

efficiency after 6-month storage.   It has been proved that the two immiscible

phases (with several discontinuous regions as shown in Fig. 1) coexisting in

liposomes not only prevent the self-aggregation of hydrophobic drugs, thereby

greatly increasing the amount of drugs held within the bilayer of the liposomes,

but also prolong the storage time without deteriorating the stability of the

5   liposomes.

[0039]   The results of Table 3 also indicated that the incorporation

efficiency of the liposomes comprising only one phospholipid (EPC or HEPC

only) were low and had dropped quickly after one-month storage, especially

the one-lipid liposomes incorporated with a high added paclitaxel/lipid ratio.

10   For example, the incorporation efficiency of the liposomes, using EPC as the

only lipid and originally having the added paclitaxel/lipid ratio of 7 mole %, had

dropped to 67.8% and 35.4% of initial incorporation efficiency after 14-day

and 1-month storage, respectively.   This result showed that the liposomes

made of one lipid didn't increase the incorporation efficiency and stability of

15   the liposomes; in contrast, the liposomes made of at least two lipids having

the said different phase transition temperatures can incorporate high content

of hydrophobic drugs and remain stable.

Table 3

Liposome Composition (molar ratio)				Paclitaxel Added Amount /Lipid	Lipid Conc.	Incorporated Paclitaxel[#]	Incorporation Efficiency (%)*				
Second Phosph o-lipid	First Phosph o-lipid	Other	Additives								
EPC	HEPC	Choleste rol	MPE G	(mole%)	(mM)	(mg/ml)	14 Days	One Month	Two Months	Three Months	Six Months

						#	*				
20	--	8	1	3	20	0.49	89.3	77.9	Dis-integrated		
20	--	8	1	7	20	0.45	67.8	35.4	Dis-integrated		
--	10	1	1	3	20	0.32	76.7	63.6	Dis-integrated		
32	8	12	2	3	40	0.77	108.4	N/A	73.9	N/A	N/A
16	3	6	2	7	20	0.92	105.3	97.9	104.6	92.2	107.0
16	3	6	2	7	40	2.02	104.4	97.3	105.0	N/A	N/A
16	3	6	2	10	20	1.34	N/A	90.0	99.5	93.3	94.8
16	3	6	2	13	20	1.60	N/A	93.7	98.5	87.5	89.4
16	3	6	2	15	20	2.07	94.2	109.7	86.0	86.1	85.1
16	3	6	2	20	20	2.90	88.7	91.7	85.5	82.2	69.0
16	3	6	2	25	20	2.32	N/A	99.6	73.3	N/A	N/A

The initial concentration of incorporated paclitaxel.

* Incorporation Efficiency = concentration of incorporated paclitaxel at the N-the day / the initial concentration of incorporated paclitaxel

Example 5

[0040]   Effects of different lipid source and lipid composition.

[0041]   Paclitaxel-liposomes were prepared along with the procedure as described in Example 1.   Hydrogenated egg phosphatidyl choline (HEPC) or hydrogenated soy phosphatidyl choline (HSPC) is selected as the first phospholipid.   Egg phosphatidyl choline (EPC) is selected as the second phospholipid, the same as Example 1.   Also, these experiments of example 5 were conducted to evaluate the influence of lipid ratio on the paclitaxel incorporation efficiency by changing the HEPC/lipid ratio or HSPC/lipid ratio.

The results are listed in Table 4.

[0042]   The results indicated that the liposomes with different particle sizes

and incorporated amounts can be obtained by adjusting the HEPC/lipid ratio.

Besides HEPC, the phospholipid from different sorts, such as HSPC, can be

5   chosen as the first phospholipid.   Soy phosphatidyl choline (SPC) is

extracted from the soy beans.   Hydrogenated soy phosphatidyl choline

(HSPC) generally has a longer chain than hydrogenated egg phosphatidyl

choline (HEPC).   Consequently, the liposomes with larger particle size can

be obtained if using HSPC as the first phospholipid.   Also, this result

10   demonstrated that the hydrogenated phosphatidyl choline (HPC) purified from

specific species could carry a high content of lipophilic (hydrophobic) drug.

However, the optimal HPC/lipid ratio with the maximum incorporation

efficiency depended on the combination of the selected phospholipids.

[0043]   In Example 5, egg phosphatidyl choline (EPC) selected as the

15   second phospholipid has a phase transition temperature of $-8\ ^{\circ}C$ which is

lower than the intravenous administration temperature ($37\ ^{\circ}C$) and the

storage temperature ($4\ ^{\circ}C$).   Hydrogenated soy phosphatidyl choline (HSPC)

selected as the first phospholipid has a phase transition temperature of 55~60

$^{\circ}C$ which is higher than the intravenous administration temperature ($37\ ^{\circ}C$)

20   and the storage temperature ($4\ ^{\circ}C$); therefore, HSPC can be combined with

the second phospholipid (i.e. EPC) to compose the liposomes.

Table 4

Drug	Second Phospho-lipid	First Phospholipid		Other Additives		Incorporation Efficiency (%)[#]	Average Particle Size (nm)
Paclitaxel	EPC	HEPC	HSPC	Cholesterol	MPEG		
0.3	8	2		1	0.5	69.2	113.3
0.3	6	4		1	0.5	63.8	120.8
0.3	4	6		1	0.5	73.6	128.4
(0.3)	8		2	1	0.5	82.2	149.5
0.3	6		4	1	0.5	62.2	167.8

Incorporation Efficiency = paclitaxel incorporated in liposome / paclitaxel added amounts.
MPEG - methoxy polyethylene glycol- distearyloyl phosphatidyl ethanolamine.

5

Example 6

[0044]   Effect of cholesterol content of liposomes.

[0045]   Varying cholesterol content affected the incorporation efficiency and

10   particle size of liposomes.   Also, incorporation of cholesterol could enhance

the rigidity of liposomes.   It is believed that the circulation time through i.v.

administration is prolonged due to the enhanced rigidity of the liposomes.

[0046]   FIG. 2 illustrates that the incorporation efficiency and the particle

sizes of the liposomes are affected by altering the cholesterol content according to a preferred embodiment of the invention.   In the general formulation shown in Figure 2, increasing cholesterol content usually reduced the incorporation efficiency of paclitaxel as well as the average particle size of

5   the liposomes.   Since the cholesterol was trapped in the phospholipid bilayer, the amounts of the cholesterol had the effect on the quantities of lipophilic drug that is also held within the phospholipid bilayer.   Accordingly, the need for cholesterol addition depends on the different combination of lipids and lipophilic drug.   In order to achieve the best incorporation efficiency and the

10   appropriate particle size of the liposomes, an optimal range of cholesterol content has to be determined first.   The results in Fig. 2 indicated that the optimal range of cholesterol content for paclitaxel-liposome system is about 0.2~0.3 in term of cholesterol/lipid molar ratio.

15   Example 7

[0047]   Storage method of the paclitaxel-liposomes.

[0048]   Paclitaxel-liposomes were stored at -20 or –75 °C after the preparation process.   Particle size and paclitaxel concentration were measured periodically.   The results indicated that paclitaxel-liposomes stored

20   at –75 °C was stable for at least 3 months.   Also, the liposomes could be lyophilized, and then stored in lyophilized cake at 4 °C for several months.

Example 8

[0049]   Incorporation of large amounts of all-*trans* retinoic acid (ATRA) in the liposomes.

5   [0050]   The liposome system of the invention can be also used for incorporating other hydrophobic drugs, such as retinoic acid.   To prepare a drug added amounts/lipid molar ratio of 1/3, 2 mg all-*trans* retinoic acid (ATRA) was added into the alcoholic admixture of 12.2 mg/ml egg phosphatidyl choline (EPC), 2.28 mg/ml hydrogenated soy phosphatidyl choline (HSPC), 10   2.28 mg/ml cholesterol, and 5.4 mg/ml methoxy polyethylene glycol-distearyloyl phosphatidyl ethanolamine (MPEG-DSPE).   The alcoholic admixture may also contain other antioxidants or cholesterol derivatives. Therefore, the composition of alcoholic admixture of this example is used for illustrating sense, rather in a restricted sense.   The solution containing ATRA 15   was evaporated under vacuum to remove solvent, and a lipid film was formed on the wall of the round-bottom flask.   After evaporation, the lipid film was hydrated with 1 ml, 10 % (w/v) of sucrose to produce suspensions of multilamellar vesicles.   Then, the liposome suspension was sonicated for 10 mins to obtain smaller unilamellar vesicles.   Retonic acid-containing 20   liposomes then were sterilized by filtration through 0.2 μm CA-membrane. Particle size was analyzed by laser particle size analyzer, and the average diameter was approximately 160 nm.   After filtration, the concentration of

retinoic acid incorporated in liposomes was determined by HPLC, and was approximately 1.9 mg/ml.   The incorporation efficiency was more than 90 %, and ATRA/lipid ratio was up to 33 mole%.

[0051]   The liposomes, prepared by the procedure described above, can

5   also encapsulate large amounts of ATRA.   Accordingly, the liposomes prepared by description of example is able to encapsulate all of retinoic acid and its derivatives.   A drug/lipid ratio range for incorporation of retinoic acid and its derivatives is 1 mole%~40 mole%, and people who skill in the art are able to use and make the same.

10

Example 10

[0052]   Incorporation of large amounts of camptothecin in the liposomes.

[0053]   To prepare a drug added amounts/lipid molar ratio of 3/10, 2 mg camptothecin was added into the admixture of 12.2 mg/ml egg phosphatidyl

15   choline (EPC), 2.28 mg/ml hydrogenated egg phosphatidyl choline (HEPC), 2.28 mg/ml cholesterol and 5.4 mg/ml methoxy polyethylene glycol-distearyloyl phosphatidyl ethanolamine (MPEG-DSPE).   The solution containing camptothecin was evaporated under vacuum to remove solvent, and a lipid film was formed on the wall of the round-bottom flask.   After

20   evaporation, the lipid film was hydrated with 1 ml, 10 % (w/v) of sucrose to produce suspensions of multilamellar vesicles.   Then, the liposome

suspension was sonicated for 10 mins to obtain smaller unilamellar vesicles.
Particle size was analyzed by laser particle size analyzer, and the average
diameter was approximately 148 nm.   Also, no visible precipitate
(crystallization of camptothecin) appears during preparation.   The

5   camptothecin /lipid ratio was up to 30 mole%.

[0054]   The liposomes, prepared by the procedure described above, can
incorporate large amounts of camptothecin.   Accordingly, people who skill in
the art are able to know that the liposomes system prepared by description of
example should be able to incorporate all of camptothecin derivative, and a

10   drug/lipid ratio for incorporation of camptothecin and its derivatives is 1
mole%~40 mole%.

[0055]   Examples 1, 2, 9 and 10 have indicated that the liposomes
prepared according to the invention can incorporate large amounts of
paclitaxel and its derivative, retinoic acid and its derivative, and camptothecin

15   and its derivative.   In accordance with this aspect of the invention, the
liposomes, which are not limited to incorporate the compounds listed above,
are capable of incorporating large amounts of paclitaxel and its derivative,
retinoic acid and its derivative, camptothecin and its derivative, and mixture of
combining two or more compounds listed above.

20

Example 11

[0056]   Toxicity of conventional paclitaxel and paclitaxel-liposomes.

[0057]   Four groups of five to six-week-old male ICR mice received

intravenous injections of either conventional paclitaxel delivery system

(cremophore EL/ethanol = 1/1) or paclitaxel-liposome delivery system at

5   doses of 20 and 40 mg/kg.   Survival rate in all the groups was recorded over

14 days, and the results were listed in Table 5.   The toxicity surveys indicated

that the conventional paclitaxel delivery system caused 20% loss of mice (1/5),

which received a dose of 20 mg/kg, within 14 days, and caused a sudden loss

of 100% of mice (5/5) which received a dose of 40 mg/kg.   However, the

10   group of mice administrated by the dose of 40 mg/kg of the

paclitaxel-liposome delivery system of the invention were survived over 14

days.   Consequently, the paclitaxel-liposome delivery system of the invention

does decrease the toxicity in comparison to conventional paclitaxel

formulation.

15   Table 5

	Dose (mg/kg)	Survival Rate
Conventional Paclitaxel delivery system	20	4/5
	40	0/5
Liposomal Paclitaxel delivery system of the invention	40	8/8

[0058]   According to the aforementioned description, the liposomes of the invention can incorporate the hydrophobic compounds such as paclitaxel in a drug/lipid ratio up to 20 mole%, and the variation of incorporation efficiency and particle size were within 15% when the liposomes were stored at 4°C over 6 months.

[0059]   In comparison with the reported formulation of paclitaxel-liposome, the drug/lipid ratio was increased to about 20 mole% by using the present liposome compositions of the invention.   The dramatic improvement resulted from the composition of two phospholipids with different physical properties. It had been discovered that two phospholipids with different phase transition temperatures created immiscible two phases, and these discontinuous regions coexisting in the liposomes could prevent paclitaxel from self-aggregation and precipitation (needle-like crystals).   Even the liposomes that contained large amounts of drugs could be maintained stable to a certain extent.   In accordance with this theory, the formulation of liposomes of the invention could be applied to incorporate other hydrophobic drugs that easily precipitated during the preparation or storage such as all-*trans* retinoic acid. According to the experiments of the invention, the liposomes of the invention increased the maximum ATRA/lipid ratio to 33 mole% while conventional formulations of liposomes had the maximum ATRA/lipid ratio of 20 mole%. Additionally, the more the difference between the phase transition temperatures of two phospholipids is, the larger quantities of the hydrophobic

drug are incorporated in the liposomes.

[0060]   While the invention has been described by way of example and in
terms of preferred embodiment, it is to be understood that the invention is not
limited thereto.   On the contrary, it is intended to cover various modifications

5   and similar arrangements and procedures, and the scope of the appended
claims therefore should be accorded the broadest interpretation so as to
encompass all such modifications and similar arrangements and procedures.